Evaluation of antibacterial potential of Graphene Oxide nanosheets and its protein functionalized form

A Thesis submitted in partial fulfilment of the requirements for the degree of Master of Technology

In

Biotechnology

By

USHA PANDEY

213BM2032

Under The Supervision of

DR. SUBHANKAR PAUL

Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela-769008, Orissa, India
2015

National Institute of Technology, Rourkela

CERTIFICATE

This is to certify that the thesis entitled "**Evaluation of antibacterial potential of Graphene nanosheets**" by **USHA PANDEY (213BM2032)** submitted to the National Institute of Technology, Rourkela for the award of Master of Technology in Biotechnology during the session 2013-2015 is a record of bonafide research work carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any Other University / Institute for the award of any Degree or Diploma.

Place: NIT Rourkela

Date: 29/5/2015

Dr. Subhankar Paul
(Associate Professor)

Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela-769008

ACKNOWLEDGEMENTS

"In order to attract more of the blessings that life has to offer, you must truly acknowledge what you already have."- **Ralph Marston**

Through this acknowledgment, I would like to express my sincere gratitude to Dr. Krishna Pramanik, HOD, Dept. of Biotechnology and Medical Engineering, NIT Rourkela for her valuable insights throughout the project.

I would like to extend my deepest gratitude to Dr. Subhankar Paul (Associate Professor, Dept. of Biotechnology and Medical Engineering, NIT Rourkela), for giving me the opportunity to do the project under his supervision, for taking keen interest and supporting me in each of my endeavours. Without his guidance and support, it would not have been possible for me to complete this research work within the allotted time period.

I would like to express my gratitude towards Mr. Deependra Kumar Ban and Mr. Sailendra Kumar Mahanta for all the technical guidance extended towards me during the course of this project work. Also I would like to thank my batchmates Prashant Kumar, Ram Sahu and R. Krishna Murthy for their precious suggestions that helped me to perform my project work in a smooth manner.

I am grateful to Dr. B. P. Nayak and Dr. Sirsendu Sekhar Ray, for collection of blood for my research purpose.

I would like to thank Dr. Anushree Roy, Prof, IIT Kharagpur and Barnita Paul for their help with Raman Spectroscopy.

Lastly, I would like to thank the non-teaching staff of Dept. of BME, NIT Rourkela for the successful completion of the project.

Date: 29/5/2015 Usha Pandey

Place: NIT Rourkela **Roll No**-213BM2029

Table of Contents

Fig. no	LIST OF FIGURES	Page no

ABBREVIATIONS

GOns	Graphene oxide nanosheets
mg	Milligram
µg	Microgram
nm	Nanometer
E. coli	Escherichia coli
P. aeruginosa	Pseudomonas aeruginosa
XRD	X-ray Diffraction
FESEM	Field Emission Scanning Electron Microscope
ROS	Reactive oxygen species
GO-LYZ conjugate	Graphene oxide lysozyme

Abstract

It is tough to develop highly effective antimicrobial agents that are not harmful to humans and do not show adverse effects on the environment as well as economically favourable. Recently Graphene oxide nanosheets (GOns) have been reported as potential candidate for having antibacterial activity. In this present investigation, GOns was synthesized by pyrolysis of citric acid and characterized by UV- Vis spectroscopy, Field emission scanning electron microscopy, Raman spectroscopy and X-ray diffraction analysis. UV-Vis absorption spectra shows the characteristic peak of GOns around 235nm and FESEM shows the fairly regular and sharp edges of GOns. XRD analysis and Raman spectroscopy shows the nanostructure of GO with characteristic peak at $2\Theta=10^{o}$ (XRD) and ~1350cm^{-1}, ~1600cm^{-1} (Raman spectroscopy). Further the toxicity of GOns and Lysozyme-GOns conjugate was assessed against human erythrocytes and the antibacterial effects were evaluated against two Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* and one Gram-positive bacteria *Lactobacillus casei*. The hemotoxcity of GOns was within 10%. Furthermore GOns reduced the growth in Gram-negative (*P. aeruginosa*) via ROS independent manner and acted as a growth enhancer for Gram-positive (*L. casei*). GOns functionalized with lysozyme increased the antibacterial effect as well as hemocompatibility. Results show that GOns has insignificant toxicity at high concentration against erythrocytes hence functionalised GOns can be safely used for antibacterial as well as biomedical applications.

Keywords: Graphene oxide nanosheets, Hemolysis, Pyrolysis, GO-functionalization, biocompatibility, Reactive oxygen species

CHAPTER 1

INTRODUCTION

Nanoparticle shows distinct properties compared to bulk material as their size approaches the nanoscale. Various methodologies has been established to synthesize a nanoparticle of definite shape and size which can be used according to its specific requirements. Graphene oxide nanosheets (GOns) attracted the huge attention due to easy availability in bulk amount, high cell compatibility, easy functionalization, and good water dispersion. These qualities makes them favourable to use as a paper like material and fillers in polymer that can be used in biological application. (Chen et al., 2008 & Zhu et al., 2010)**.**Nanoparticles having small size have various properties which offers many new developments in the fields of biosensors, biomedicine, and bio nanotechnology.

Nanotechnology is also being utilized in medicine for diagnosis, therapeutic drug delivery and the development of treatments for many diseases and disorders. Graphene is a two-dimensional material having high mobility and high carrier concentration and has been used for many fields and applications, such as field effect transistors, transparent conducting electrodes, supercapacitors, batteries, photocatalysis, gas sensors, field emission devices, and nanocomposites (Lin et al., 2011 & Schedin, et al., 2007).

Other nano particle or antibacterial agents have biocompatibility problem and high economic cost hence Graphene oxide is a best alternative. Graphene oxide can be used in various applications such as photothermal therapy of cancer, gene transfection, and magnetic resonance imaging. (Yang, et al., 2010, Feng et al., 2011 & Cong et al., 2010).Organic antibacterial agents having many problems such as low heat resistance, high decomposability, and short life expectancy so inorganic agents can be used because they are stable at higher temperature and safe hence nanostructured material are mostly investigated having antibacterial properties based on size, surface properties and structure (Fang *et al*., 2006, Jung et al., 2008, Raghupathi, et al., 2011).Chemically modified graphene (CMG) due to its best electrical, mechanical, and

thermal properties has different application, such as polymer composite, sensors and biomedical. Chemically modified graphene oxide which is generally synthesized from graphite oxide has a simplest way to produce CMG platelets. GO is an atomically thin sheet of graphite that covalently decorated with oxygen-containing functional groups, either on the basal plane or at the edges. Due to edge effect and quantum confinement GQDs size (100 nm) shows distinct optical and electronic properties. (Li et al., 2008).Graphene oxide consists of reactive oxygen functional groups through chemical functionalization which makes it potential material to use in various application. (Geim et al., 2007 & Park et al., 2009).

GO is usually synthesized by chemically exfoliating graphite. To synthesize quantum dots, ‘‘top–down’’ and ‘‘bottom–up’’ method have been established. Top down methods which uses high-resolution electron beam lithography for carving graphite. (Ponomarenko et al., 2008) and hydrothermal method (Shen et al., *2012*) has some disadvantages and these methods requires special equipment and there is a problem in maintaning the size distribution of products. Bottom–up strategies where organic precursors are carbonized by thermal treatment (Yan et al., 2009 & Liu et al.,2011), which allow minute control over the morphology and the size. Here we have investigated a simple bottom–up method to synthesize GO by carbonization of organic precursor, citric acid (CA).Some Oxidative stress caused by graphene based materials, one may be due to Reactive oxygen species (ROS) where oxidative stress is induced by ROS generation by GO and the other possible way is ROS-independent oxidative stress, in which graphene-based materials disturb or oxidize a vital cellular structure which may causes the disruption of a specific microbial process without ROS production. (Zhang et al., 2010 & Lyon et al., 2010). Graphene oxide has differential toxicity toward Gram-negative bacteria compared to Gram positive bacteria because of different nature of cell wall and Gram-positive bacteria possess a thick peptidoglycon layer compared to Gram-negative bacteria. (Hu et al., 2011 & Akhavan et al., 2010). In recent study it was shown that bacteria grew faster at GO

concentration of 25 μg/mL than cultures without GO. (Ruiz ON et al., 2011).Cell damage of *P. aeruginosa* might be due to either oxidative stress or physical disturbance. In the recent study it was found that after exposure to dispersed GO most *E. coli* cells became flat and lost their cellular structure. (Hu et al., 2010 & Liu et al., 2011). The main reason behind effective direct interaction of Graphene oxide with microorganism is their high aspect ratio which is due to its nanostructure. Due to problems in the synthesis of Graphene nanowalls the toxicity of graphene sheets against bacteria by interacting with its very sharp edges has not been reported. Based on the work to be done till now it was found that graphene papers are biocompatible compound and the growth of *E. coli* bacteria can be inhibited by Graphene oxide but with a low cytotoxicity. (Chen et al., 2008 & Hu et al., 2010)

GO is having toxicity to human fibroblast cells which is dose dependent and when the dose of GO is increased upto 50 μg/mL it causes cytotoxicity. On the other side, other investigation shows good biocompatibility of GO where GO enter inside the A549 cells and showed no toxicity unconcerned of the size or dosage of GO. (Ryoo et al., 2010 & Chang et al., 2011). Whenever proteins binds to planer surfaces it induces some changes in the secondary structure but due to large curvature of nanoparticle the secondary structure of protein is retained. It is reported that in any conjugation process there is always some kind of perturbation in the protein structure to an extent. ZnO and Au NPs are biocompatible as well as inert hence they are mostly used nanoparticle. Au NPs are used in drug delivery for DNA and protein detection. When lysozyme is adsorbed onto silica NPs surface there is a change in the conformation of secondary structure and tertiary structure. (Vertegel et al., 2004; Wu and Narsimhan et al., 2008; Bhattacharya et al., 2007). In recent investigation it has been found that whenever protein is adsorbed onto nanoparticle's surface there is increment in turns and sheet but loss in α helical structure which is observed in circular dichroism spectroscopy. In this paper we have investigated the antibacterial behavior, hemocompatibility of GO and GO-LYZ but there is still

some research should be done to evaluate the behavior of GO against other bacteria. It was observed that when GO was used without protein it killed the significant amount of cells when interacted with the cells. The cytotoxic effect was largely diminished when GO was precoated with FBS and put in the medium with 10% FBS. 100 % cell survival was found at 20 μg/mL concentration of GO coated by FBS and corresponding similarity were seen with higher incubation times and GO which was coated by BSA. These reports suggested that the cytotoxicity of GO was due to straight interactions of GO with cells. From the investigation it was found that BSA may not be the principal constituent for the production of a protein corona over the nanomaterials. (Cedervall et al., 2007 & Dell'Orco et al., 2010).

The imapact of Graphene which was functionalized with amine (G-NH2) has not been reported for biomedical use, while single-walled carbon nanotubes modified by amine were known to be cytoprotective toward neuronal cells. (Lee et al., 2009 & Lee et al., 2011).Biological modification of GO improves its biocompatibility, solubility and selectivity. Hence lots of research has been done on graphene modification and functionalization (Allen et al., 2010). Health and environmental effect of Graphene oxide should be investigated before its application. ROS assay was performed to check whether death is ROS mediated or not. GO and GO-LYZ conjugate was characterized by Field emission scanning electron microscope (FESEM) UV-vis spectroscopy, X-ray diffraction (XRD) and Raman spectroscopy. Through this study we compared the antibacterial activity of GOns against *E.coli, P. aeruginosa and L. casei* and evaluated the hemocompatibility against erythrocytes. GO showed less cytotoxicity against erythrocytes hence is biocompatible and act as an antibacterial agent for *P. aeruginosa*. GO surface was modified by the Lysozyme and its antibacterial effect and hemocomaptibility was also evaluated and found to be biocompatible and showed enhanced antibacterial effect. Hence in future GO can be used as a potential antibacterial agents and as a drug carrier.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Nanoparticle and their application

Nanoparticles are used in various places such as electrical, biological textile and chemistry where crucial role is played by shape and size of colloidal metal particles and this property can be used in many application such as preparation of magnetic, electronic devices and bio composites. Optical, catalytic and electromagnetic properties of metal colloids depends on size and shape of the particles. The physical and chemical properties of material can be changed when material is prepared in a very small size particle. In nano-dimension due to increment in percentage of surface molecule in comparison to bulk molecule the action of the particle in nano dimension is enhanced therefore, the properties of the particle like heat treatment, mass transfer, catalytic activity, etc. are all increases. Metal nanoparticle has more industrial application compared to non-metal. Nanoparticle can be used as a biosensor, antibacterial agents, drug delivery agents and cancer treatments agents.

The removal of bacteria from water and sanitation system is required in order to overcome water borne diseases. (Li et al., 2008 & Gangadharan et al., 2010).Many chemical disinfectants such as free chlorine, chloramines, and ozone which are used in the water industry mostly are carcinogens. (Kratschmer et al., 1990). Furthermore, the impendance of microbes to these usual chemical disinfectants is increasing; other options are hence is needed. A number of nanoparticles such as Ag, Cu, ZnO, and TiO2, show great toxicity to a large number of pathogens and have been studied as antibacterial agents (Zhang et al., 2009 & Ruparelia et al., 2008)**.**

2.2 Synthesis of GO nanosheets (GOns)

GO can be produced by various methods such as hummers method, citric acid pyrolysis method, modified hummer's method, fast and facle method, brodie method etc. Among all the methods citric acid pyrolysis method was followed due to easy availability of chemical, less time consuming and formation of Graphene oxide nanosheets.

2.3 Application of GOns

Pristine carbon nanotubes can't be used due to its remarkable cytotoxicity to mammalian cell and acid treated surface functionalization. Another factor is due to its high economic cost (Lin et al., 2009 & Yuan et al., 2008).Graphene oxide attracted the huge attention due to easy availability in bulk amount, high cell compatibility, easy functionalization, and good water dispersion. These qualities makes them favourable to use as a paper like material and fillers in polymer that can be used in biological application. (Chen et al., 2008 & Zhu et al., 2010).Graphene paper is a biocompatible substrate for adhesion and proliferation of L-929 cells, 20 neuroendocrine PC12 cells, oligodendroglia cells, and osteoblasts. (Agarwal et al., 2010).Graphene oxide can be used as a carrier to transport water-insoluble drugs into cells (Sun et al., 2008 & Liu et al., 2008).Nanoparticles can be used in both passive and active targeting strategies which can enhance the delivery of drugs to the cancer cells without any side effects to normal cells. (Maeda et al., 2001 & Allen et al., 2002). Biological modification of GO improves its biocompatibility, solubility and selectivity. Hence lots of research has been done on graphene modification and functionalization (Allen et al., 2010)

Nanoparticle can be beneficial or detrimental after increasing its biological activity. Various nanoparticle can reach up to lungs, skin and brain. (Koziara et al., 2003; Oberdorster et al.,

2004).GO has good biocompatibility yet there are various reports where it is found that GO is toxic to cell at large concentration. GO at 50µg/mL and higher concentration is toxic to human fibroblast cells. (Agarwal et al., 2010; Hu et al., 2010; Wang et al., 2010).It was reported that dose-dependent oxidative stress was found in A549 cells by GO but it can't damage the cell membrane. (L. Wang et al., 2011). It was investigated that apoptosis or necrosis could be induced by GO. To observe the two mechanism in PC12 cells enhanced by Graphene sheets, two important enzymes were measured. Caspase activation indicating apoptosis and Lactate dehydrogenase (LDH) release necrosis. In reports sufficient LDH leakage was measured after 24 h. at GO 100 µg/ml concentration. (Nel et al., 2006 & Shi et al., 2006).

2.4 Antibacterial potential of GOns

It has been investigate that horseradish peroxidase (HRP) and lysozyme continuously adsorbed on GO because the GOns sheet is filled with oxygen-containing groups, which makes it possible to immobilize enzymes without any surface manipulations or coupling reagents (Zhang et al. (2010). Ag NP/GO composite are expected to show efficient antibacterial activity towards both Escherichia coli and Staphylococcus aureus. The efficient impact of this work may be its use as a microbicide for shut down of pathogens in water, or as a new component for killing microorganisms that may cause biofouling in downstream filters. (Bao et al., 2011).

Graphene-based paper consists of flexibility as well as mechanical stiffness. A recent investigation showed that bacteria did not increase on independent paper made up of a Tween/ rGO composite. (Park et al., 2010).The antibacterial mechanism of GO leads to membrane stress which is enhanced by sharp edges of graphene nanosheets that may result in substantial damages on cell membranes which leads to loss of bacterial membrane structure and the

leakage of RNA.(Akhavan et al.,2010). Oxidative stress caused by graphene based materials may come from several ways, one is Reactive oxygen species (ROS) mediated oxidative stress where oxidative stress is induced by ROS generation by GO and the other possible way is ROS-independent oxidative stress, in which graphene-based materials disturb or oxidize a vital cellular structure which may causes the disruption of a specific microbial process without ROS production. (Zhang et al., 2010 & Lyon et al., 2010)

GO is generally toxic to bacteria and on adding PVK in GO leads to an enhanced dispersion of GO in the solution giving a greater aspect ratio that leads to an enhanced interaction with the bacteria and higher toxicity. (Santos et al., 2011). Recent investigation have shown that graphene-based nanomaterials shows killing effects on a various kind of microbes such as the Gram-negative bacteria *E. coli* and P. *aeruginosa* and the Gram-positive bacteria S. aureus. (Das et al., 2011 & O. Akhavan et al.,2010).Cell wall membrane of bacteria can be damaged by Graphene nanowalls due to very sharp edges of the nanowalls which causes straight contact interaction of bacteria. The growth of *Escherichia coli*. (*E. coli*) bacteria can be inhibited by GO suspension but with a negligible cytotoxicity, and photoinactivation of *E. coli* bacteria can be enhanced by graphene sheets on surface of a graphene/TiO2 composite thin film.(Akhavan et al. , 2010, Hu et al., 2010 & Akhavan et al., 2009).

A recent investigation has shown that contact of GO with *E. coli* and *Staphylococcus aureus* can cause reductions in the growth about 51 and 61%, respectively. It was noted that growth inhibition zones in *E. coli* and *S. aureus* was found when graphene paper was used. (Bao et al., 2011).It was reported that when GO was kept in the center of a nutrient media plate which is already having bacteria, zone of growth inhibition was not formed. (Das et al., 2011).Fully distributed GO sheets shows the efficient antibacterial effect and on the other side aggregated

GO sheets in LB (Luria−Bertani) medium act as a cell growth activator. (Liu et al.,2011 & Ruiz et al.,2011).

Graphene oxide shows antibacterial effect against E.coli which results in disruption of cell wall that can be caused by either substancial disruption or oxidative stress. Graphene oxide has differential toxicity toward Gram-negative bacteria compared to Gram positive bacteria because of different cell walls and Gram-positive bacteria possess a thick peptidoglycon layer compared to Gram-negative bacteria. (Hu et al., 2011 & Akhavan et al., 2010).In recent study it was shown that bacteria grew faster at GO concentration of 25 μg/mL than cultures without GO. (Ruiz ON et al., 2011).Cell damage of *P. aeruginosa* might be due to either oxidative stress or physical disruption. In recent study it was found that after exposure to dispersed GO most *E. coli* cells become flattened and lose their cellular integrity. (Hu et al., 2010 & Liu et al., 2011).

2.5 Lysozyme

Fleming found a compound which was strong bacteriolytic and can disrupt the thick layer of bacteria and it was similar to ferments so he named it Lysozyme.Lysozyme was found in tears mucus, saliva, blood serum and plasma. It is found in egg white and turnip also.Lysozyme is basic in nature and its iso -electric point at pH 10.5-11 and its molecular weight is 14722.There is high content of arginine and absence of sulfhydryl group. (Fleming et al., 1922 & Fevold et al., 1951).Lysozyme is an antibacterial enzyme which can hydrolyse the peptidoglycan layer of the gram positive bacterial cell wall. (Jolles, 1964).From earlier studies it was found that the lytic activity of Lysozyme depends on the pH, temperature and sodium concentration. (Fleming

et al., 1922). Lysozyme activity depends on pH and optimum at 6.5 and maximal at ionic strengths between 0.1-0.2. (Smolelis et al., 1952)

2.6 Study of Protein-GO nanosheets interaction

Proteins are more adhesive to solid surface because it consists of hydrophobic and hydrophilic patches on their surfaces. GO has basal planes where protein can be adsorbed stably. Due to presence of Tyr residues bovine serum albumin (BSA) is good reductant. BSA is reductant as well as stabilizer for the preparation of BSA-GO conjugates. (Hlady et al., 1996 & Basu et al., 2008).GOS and BSA conjugation is done by diimide-activated amidation method. When conjugation is over the GO nanosheets is fully exfoliated. Twenty percent BSA is there in GOS-BSA conjugate which shows high efficiency of covalent functionalization. (Jianfeng Shen et al., 2010)

Whenever proteins binds to planer surfaces it induces some changes in the secondary structure but due to large curvature of nanoparticle the secondary structure of protein is retained. It is reported that in any conjugation process there is always some kind of perturbation in the protein structure to an extent. ZnO and Au NPs are biocompatible as well as inert hence they are mostly used nanoparticle. Au NPs are used in drug delivery for DNA and protein detection. When lysozyme is adsorbed onto silica NPs surface there is a change in the conformation of secondary structure and tertiary structure. (Vertegel et al., 2004; Wu ,Narsimhan et al.,2008; and Bhattacharya et al., 2007).In recent investigation it has been found that whenever protein is adsorbed onto nanoparticle's surface there is increment in turns and sheet but loss in α helical structure which is observed in circular dichroism spectroscopy.

CHAPTER 3

MATERIAL & METHODS

3.1 Material

Citric acid was taken from Himedia laboratories Pvt. Ltd., Mumbai, India. Pure chemicals were used in all methods including synthesis of GO nanoparticles, media preparation for growing bacterial cells. The bacterial cultures of *E.coli, P. aeruginosa,* were taken from Life science lab NIT Rourkela. All glass wares (Conical flasks, Measuring cylinders, Beakers, Petri plates and Test tubes etc.) were taken from borosil, India.

3.2 Preparation of GO nanosheets (GOns)

GOns was prepared by pyrolysis of citric acid. 2 g of Citric acid was taken and put into10 mL glass tube and heated to 200 ºC using a heating furnace. The Citric acid was liquated after 5 min and the liquid was converted from colorless to pale yellow and after 30 min color was changed to orange which shows the formation of GQDs. On continuing the heating orange liquid would finally covert to black solid within 2 h which suggests the GO has been formed. The orange liquid for the preparation of GQDs was added drop by drop into 100 mL of 10 mg/ mL NaOH solution, under fast stirring. After this pH was neutralized to 7.0 with NaOH and the aqueous solution of GQDs was obtained. The black solid was diffused with 50 mL of 10 mg / mL NaOH solution, and again neutralized with the same concentration of NaOH and aqueous solution of GO was obtained.

3.3 Preparation of GO-Lysozyme (GO-LYZ) binary nanoconjugate

1mg/ml of GO from stock of 14.4mg/ml Graphene oxide to the final volume of water 4ml was added and stirred for 5 min at 300 rpm. After that 1mg/ml of lysozyme nanoparticle from

25mg/ml of stock was added and kept for stirring for 8th hr. Centrifuge was done at 20000g for 15 min. Pellet and supernatant was separate out. Pellet was dissolved in 1ml of water and OD was taken at 200-800 nm scan range. Graph was plotted.

3.4 Characterization techniques

The GO nanosheets (GOns) were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda35.The scanning range for the samples was 200-800 nm. The spectrophotometer was equipped with "UVWinlab" software to record and analyze data. By taking a blank reference base line correction of the spectrophotometer was done. The UV-Vis absorption spectra of all the samples were taken and data were plotted in origin 8.Floroscence emission spectra was observed in Perkin-Elmer LS55 fluorescence spectroscopy and the scan rate was 300nm/min. The crystalline morphology of Graphene oxide was studied by X-ray diffraction technique. The GO samples were scanned in the 2θ ranges 5º to 40º in continuous scan mode. The scan rate was 10º/min. NOVA NANOSEM transmission electron microscope machine was used to characterize shape and morphology of nanoparticles. GOns sample was sonicated and 100µg/ml of GOns solution was prepared and minute drop of this sample (10µl) was placed on glass slide and allowed to dry and same was done for conjugates. Slide was kept for drying in LAF for 2 hour and sample was kept in respected holder and FESEM image was obtained. Raman spectroscopy (Horiba JY, France) was done to confirm the synthesis of GOns and Laser of 488nm wavelength was used.

3.5 Antibacterial assay

3.5.1 Growth inhibition in liquid medium

The antibacterial action of GOns and GO-Lysozyme conjugate both in liquid LB growth medium and on agar plates were investigated. *E. coli* culture was grown in Luria broth (20 gm. in 1000 ml distilled water). Cold *E. coli* cells were grown overnight in the LB broth to synthesize inoculum. The bacteria cultures grew in shaking incubator at 37ºC and 130 rpm. GOns was distributed in autoclaved deionized water. Aqueous distribution of GO nanoparticles of desired concentration was produced. Fresh bacterial grown inoculums of *E .coli, P. aeruginosa. L. casei was* incubated in the presence of GOns with concentration of 0, 50, 100,300 and 500µg/ml that added in each glass tube to evaluate the bacterial cell growth pattern at 37ºC and 130 rpm. Similarly GO-Lysozyme nanoparticles conjugates of same concentration added in each beaker to evaluate the bacterial cell growth pattern under the exact condition. Total solution volume which was used in each beaker was 5 ml. In liquid medium, growth of *E. coli* was found by measuring optical density (OD).Optical density measurements of the samples were carried out at λmax =600 nm against growth media control by UV-Vis spectroscopy after every 2 h time interval and up to 24 h for preparing growth curve and same for other bacteria. Control flask obtained 5 ml of all the initial reaction components except the nanoparticles and apart of that we took OD at three different hour 8^{th}, 16th, 24^{th}.

3.5.2 Well plate assay

The antibacterial assays were also done by standard well plate method. Luria broth agar was used to cultivate bacteria. The agar was autoclaved and cooled. Agar media was poured in

petridis and allowed for solidifying. After solidifying the bacterial inoculum (100µl) was spread over the Petridis and kept it for 10 min for drying. Well was made by using 1ml tip and respected concentration of GOns was added in the well. Petridis was kept at 37ºc and 130rpm for overnight and the next day colony was obtained.

3.5.3 Cell viability assay

In this cell viability assay GOns treated sample was taken after16th hour and centrifuged at 5000rpm for 10min and this is repeated for three times till the media is finished. Sample and tryphan blue in (1:1) was added in the Heamocytometer slide and the dead cell was counted in the box.

3.6 ROS assay

In this assay N acetyl cysteine (NAC) (2mM) was added in the sample where GOns is already given. After adding NAC the sample was kept at 37ºC and 130 rpm. The next day at 16th hour the OD measurement was taken at 600nm.

3.7 Hemolysis assay

Blood was collected in tubes. Erythrocyte was washed two times with 10 times volume of free pyrogen free saline (0.9% NaCl) and centrifugation was done at 1000g for 10min.Erythrocyte pellet was gently resuspended with pyrogen free saline and Erythrocyte was diluted upto 0.8%.four ml of cells were incubated with 5, 10,20,40,80,100 µg/ml of GOns for 1hr.Erythrocyte with saline act as negative control and Triton X as positive control. After one hour of incubation OD was taken at 404nm.

CHAPTER 4

RESULT & DISCUSSION

4.1 Synthesis of graphene oxide nanosheets (GOns) by pyrolysis of citric acid method

GOns was synthesized according to the method given in the 'Material and Methods' and the colour of GOns was found brown which shows the complete carbonization of citric acid.

Figure 1: Synthesised GOns by pyrolysis of citric method.

4.2 Characterization of GOns

4.2.1 UV–Vis spectral analysis of GOns

This figure shows the UV-visible absorption spectrum of the suspened GO nanosheets. UV-Vis absorption spectra of GO shows the broadening of peak around 230-235nm. The GO shows a broad UV–Vis absorption below 600 nm without any significant peak which suggests that sp2 clusters present in GO is not homogeneous in size. The broad absorption peak at about 230 nm suggests the π- π* transitions of aromatic C–C bond, and a shoulder peak at about 300 nm shows the π- π*transitions of C=O bonds.

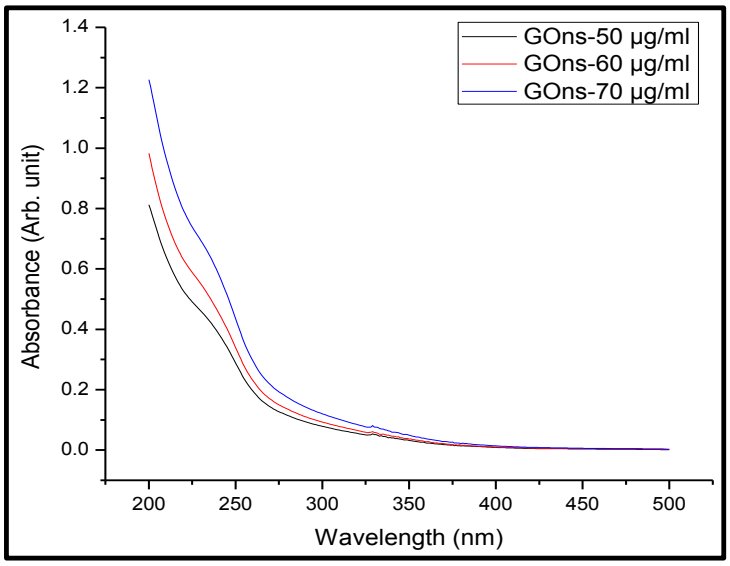


Figure 2: UV-Vis absorption spectra of the GOns.

4.2.2 X-ray diffraction pattern of GOns

XRD analysis is used to find the average crystalline properties of the GO sheet. GO nano sheets (GOns), synthesized by citric acid pyrolysis process gives peak around 2Θ=10º which is very less compared to graphite (2Θ=26º) and shows complete oxidation of graphene.The interlayer spacing between graphene was found between 0.85 to 1nm.

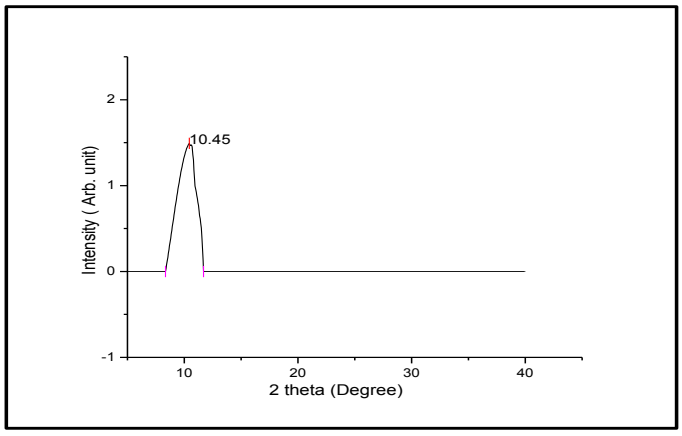
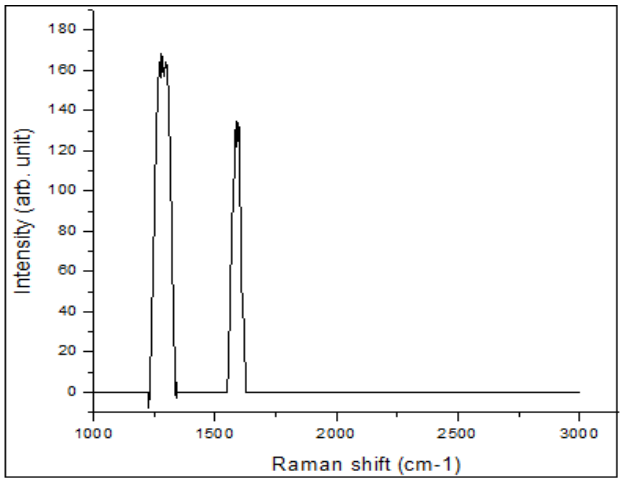


Figure 3: XRD pattern of the graphene oxide nanosheets (GOns).

4.2.3 Raman spectroscopy of GOns

Raman spectra analysis of GO nanosheets shows a band of high intensity at ~1581 cm-1 (G band) and a weak band at ~1340 cm-1 (D band). The G band shows the first-order scattering from the E2g phonon of sp2 carbon bonding and D bands structural deformity respectively and these values tells about the symmetry which are generally given to the graphite structure and local deformity mainly situated at the edges of graphene and graphite platelets, respectively.



\ **Figure.4:** Raman spectra for the GO nanosheets.

4.2.4 FESEM analysis of GO nanosheets (GOns)

The morphology of Graphene oxide nanosheets was studied by FESEM which shows the irregular shapes and sharp edges of GOns between 100-500nm ranges. After sonication the size of GOns is reduced and increasing the sonication time leads to reduction in the size of GOns.

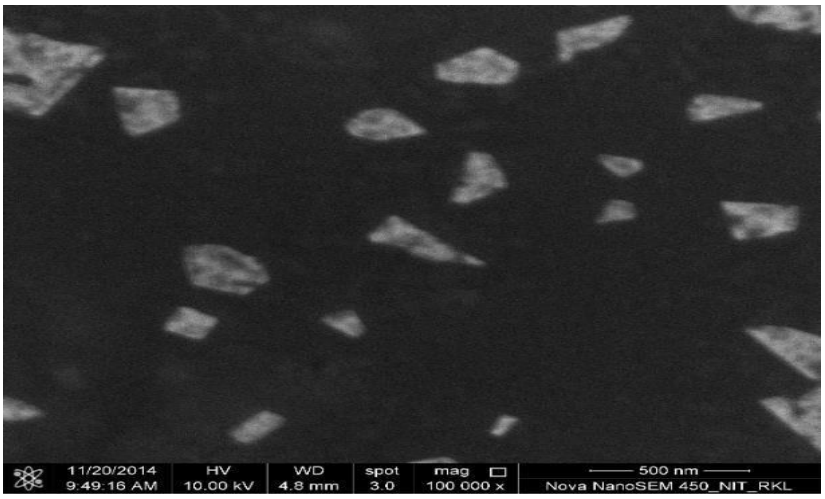


Figure 5: FESEM image of graphene nanosheets (GOns).

4.3 Antibacterial assay

4.3.1 Well plate assay GOns

GOns was given at 40,120,360 and 1080 µg/ml. For *E.coli* and *L. casei* no zone of inhibition was found. It might be the reason that GOns is non-diffusing in agar or GOns is nontoxic at this concentration.

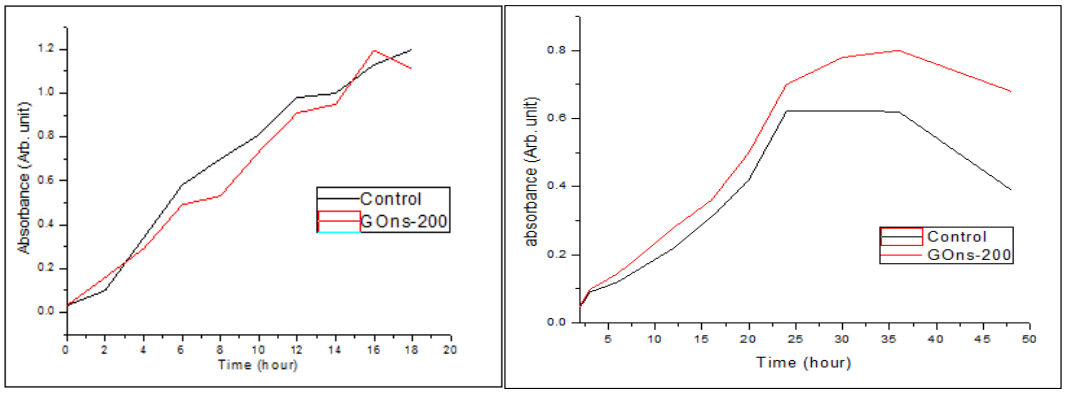
(a) **(b)**

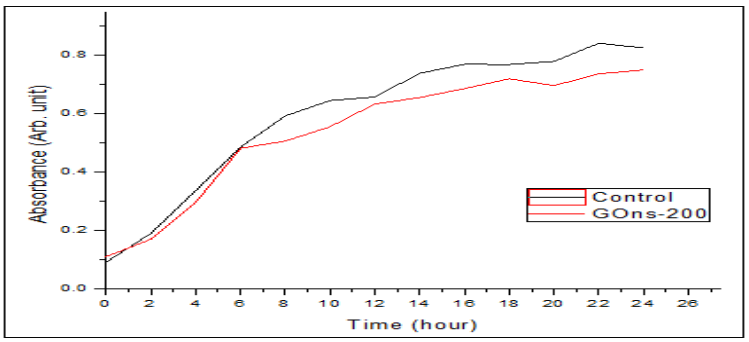
Figure6: **(a)** *L. casei* (a) treated with 40,120,360 and 1080 µg/ml of GOns and *E.coli* (b).

4.3.2 Effect of GOns in the growth curve of *E.coli, P. aeruginosa and L.casei*

The *E.coli* growth curve was reduced at 200 µg/ml of GOns compared to control.This reduction is concentration dependent and it can be due to the sharp edges of GOns which helps in rupture of the bacterial membrane. The growth curve of *P. aeruginosa* is also reducing. *L. casei* growth curve is shifting above the control which is due to the biofilm formation on the GO by bacteria which is acting substrate for bacteria and enhances its growth.

(a) (b)





(c)

Figure 7: Growth profile of (a) *E.coli (*b), *P. aeruginosa* (c) *L.casei* (c).

4.3.3 Time and Concentration-dependent Antibacterial Activity of GOns

GOns shows the mild cytotoxicity in *P. aeruginosa* and *E.coli* but in *L. casei* the cell growth is enhanced in concentration dependent manner. In *P. aeruginosa* reduction in OD is concentration and time dependant which is due to sharp edges of GOns which interacts withe the membrane of *P. aerugiosa* and disrupt it but in case of *L. casei* the same GOns is acting as growth enhancer because GOns is acting as a substrate for the growth of *L. casei* forming biofilm on graphene oxide nonosheets and grew faster

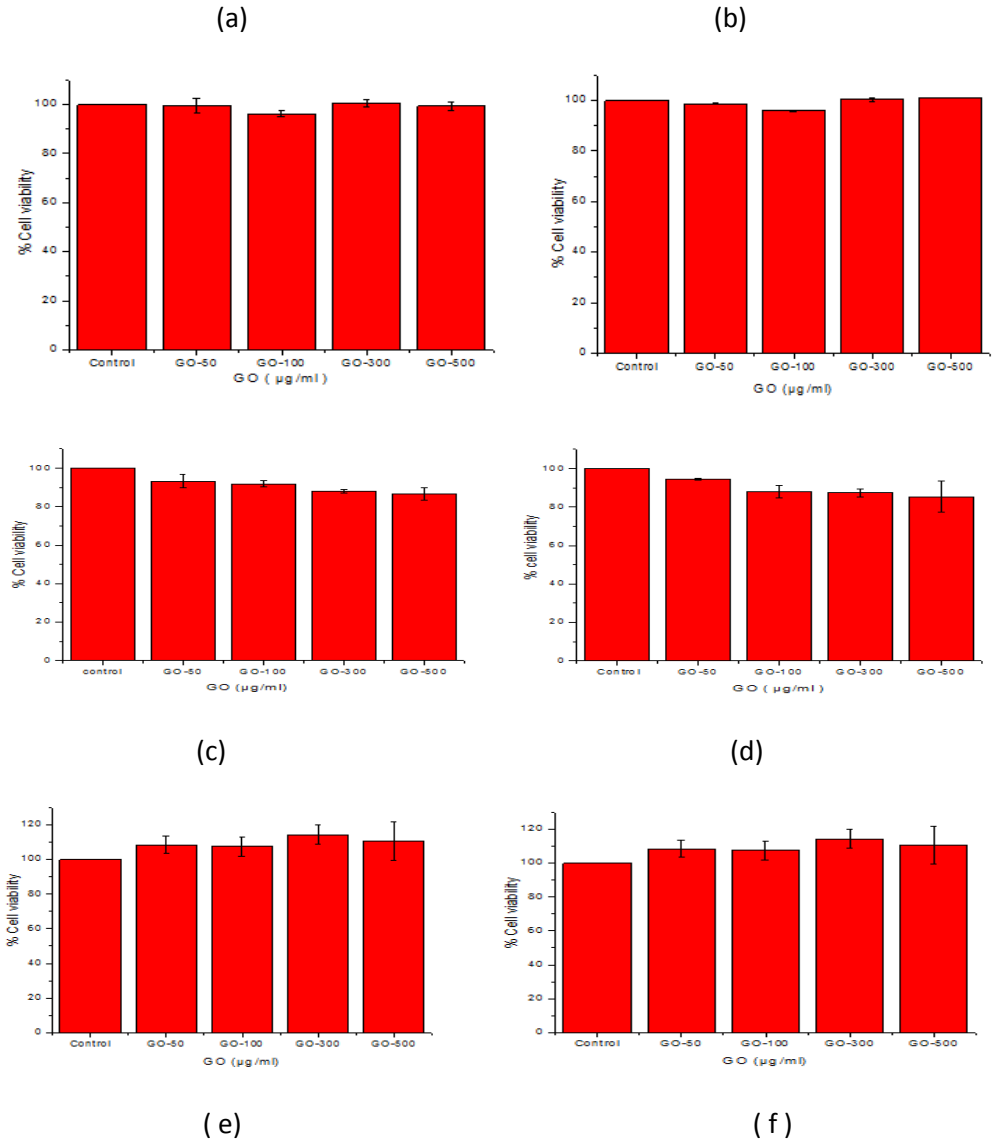


Figure. 8: Cell viability of *E.coli (a, b*), *P. aeruginosa (c, d)* and *L. casei* (e, f) treated with GOns 50,100,300 and 500 µg/ml at 16th h.

4.3.4 FESEM analysis of GOns treated bacteria

FESEM images of bacteria interacted with different concentration of GOns after 16th hr. was taken. It was shown that at increasing concentration of GOns in both the Gram-negative

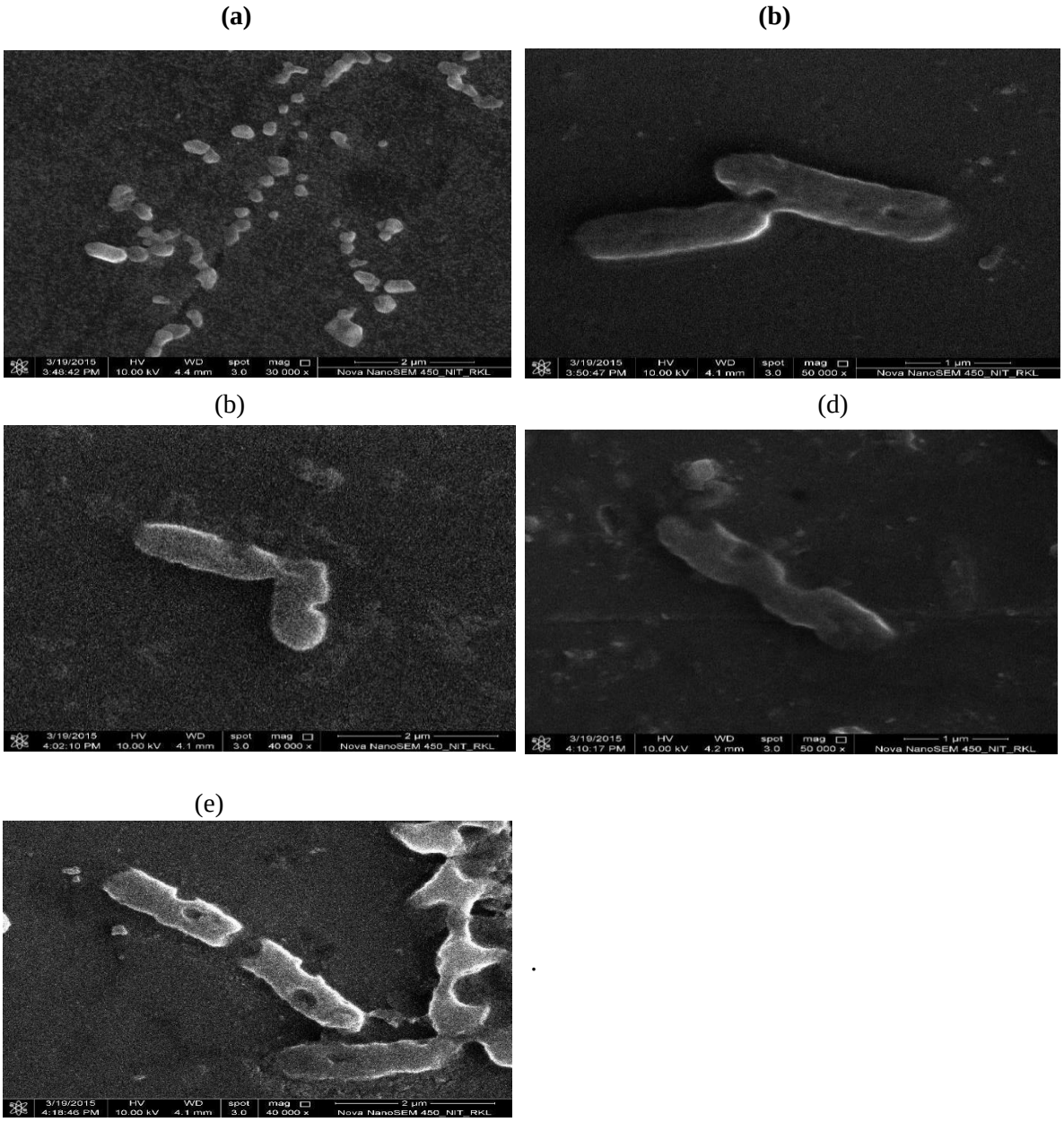


Figure 9 : *E.coli* cell without GOns **(a)** and treated with different concentration of GOns 50µg/ml **(b)** 100 µg/ml **(c)** 300 µg/ml **(d)** and 500 µg/ml **(e)** at 16thh.

bacteria the membrane rupture was increased and reached maximum at 500µg/ml of GOns but in *L. casei* there was no membrane rupture was observed even at high concentration. Due to sharp edges of GOns it ruptures the membrane of gram negative bacteria but act as a substrate for the growth of *L. casei* and no membrane rupture was seen in *L. casei*.

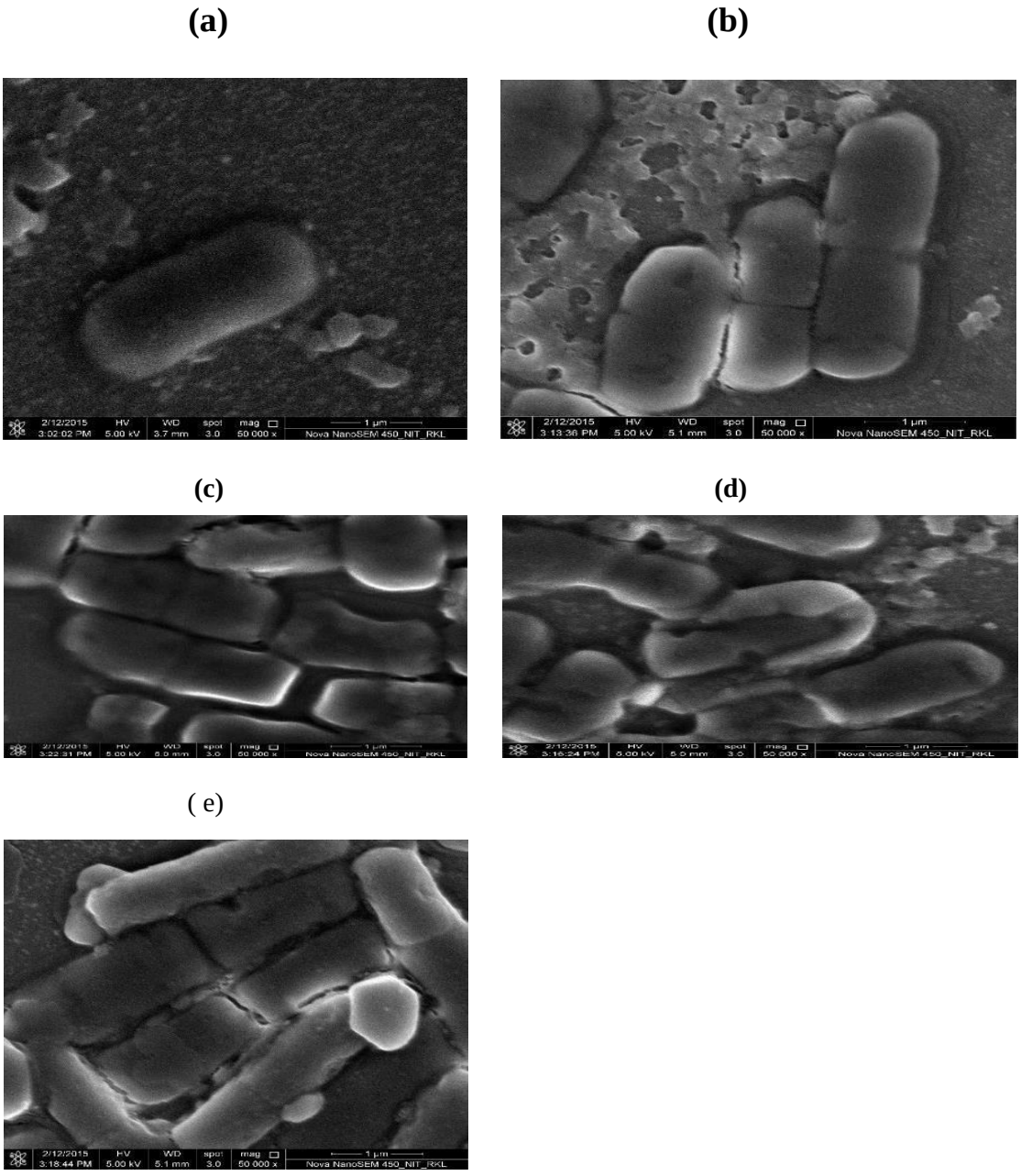
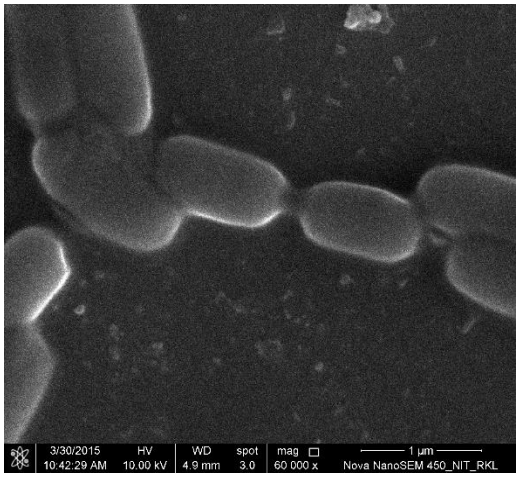
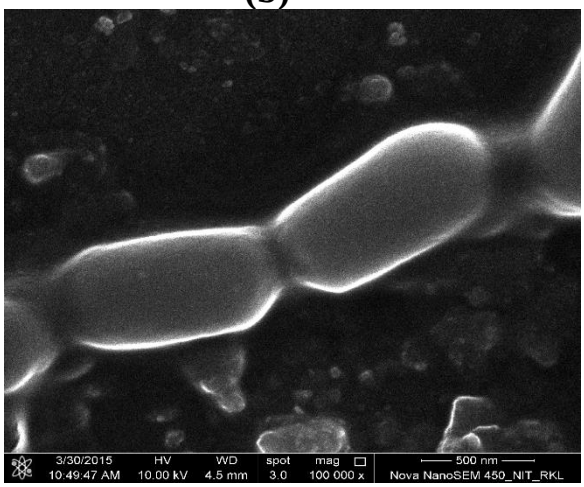


Figure 10: *P. aeruginosa* cell without GOns and treated with different concentration of GOns 50µg/ml **(b)** 100 µg/ml **(c)** 300 µg/ml **(d)** and 500µg/ml **(e)** at 16thh.

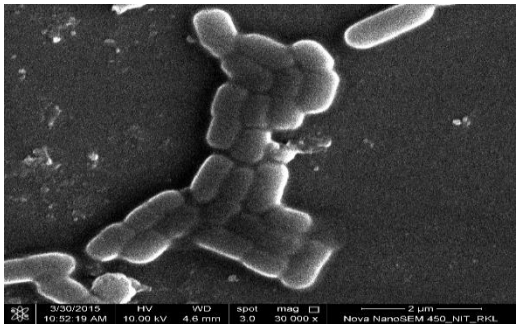
(a)



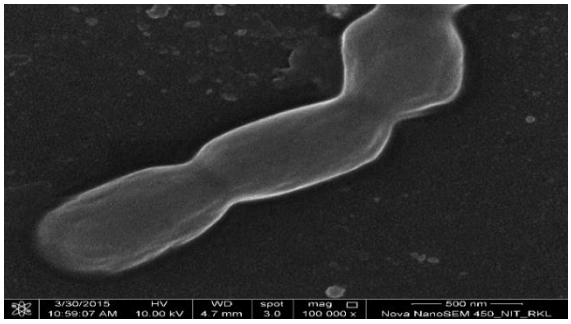
(b)



(c)



(d)



(e)

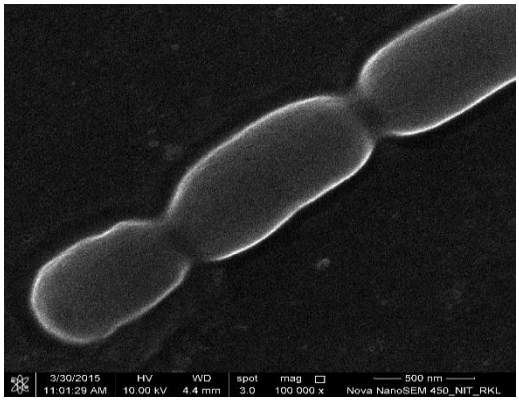


Figure 11: *L. casei* cell without GO **(a)** and treated with different concentration of GO 50µg/ml **(b)**100 µg/ml **(c)** 300 µg/ml **(d)** and 500 µg/ml **(e)** at 16^{th} h.

4.4 Hemolysis assay of Graphene oxide nanosheets (GOns)

Gons shows hemolysis as concentration of GOns is increased. Maximum hemolysis was 10% as compared to triton X 100.The membrane of Erythrocyte was ruptured by GOns in dose dependent manner which leads to release of free hemoglobin in supernatant. This result indicating that the damage of the Erythrocyte membrane might be caused by high electrostatic connections between negatively charged oxygen groups on the GOns surface and positively charged phosphatidylcholine lipids which are situated on the Erythrocyte outer membrane.

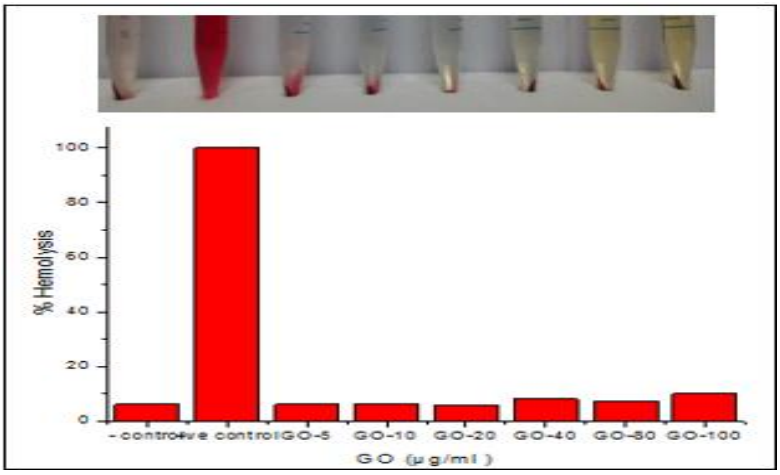


Figure 12: Hemolysis image of Erythrocyte treated with GOns and error was within 3%.

4.5 ROS assay

P.aeruginosa was given different doses of GOns, 50,100,300 and 500µg/ ml. In one tube GOns was given with N Acetyl Cysteine (NAC) and another tube GOns was given without NAC and reduction in OD was found on increasing the concentration of GOns. There is no ROS generation mediated death of cell because there is no increment in OD in tubes (with NAC) compared to tubes (without NAC) at 16th hour. This death may be because of straight interaction of sharp edges with bacteria where ROS production is not taking place.

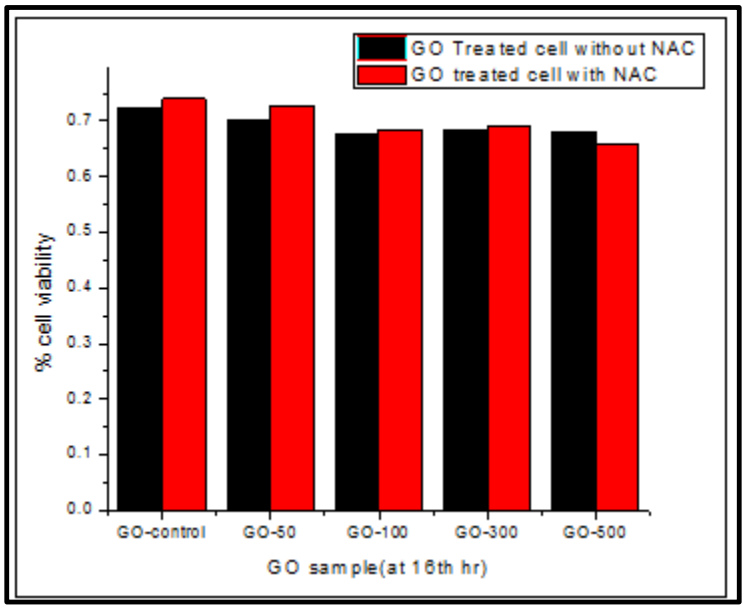


Figure13: ROS assay of GO treated *P. aeruginosa* and error was within 3%.

4.6 In vitro interaction of GOns nanosheets and Lysozyme monitored by spectroscopic techniques

4.6.1 UV-Vis spectral analysis of GO-LYZ conjugate

GO-Lysozyme (GO-LYZ) conjugate peak was found around 230nm.Lysozyme peak we get around 280nm.Peak shift is the confirmation that GO-LYZ conjugate has been formed. Lysozyme get adsorbed on the surface of the GO and amino group of protein react with the carboxylic group of GO by the help of glutaraldehyde which makes conformational changes in the protein and peak shifts occur.

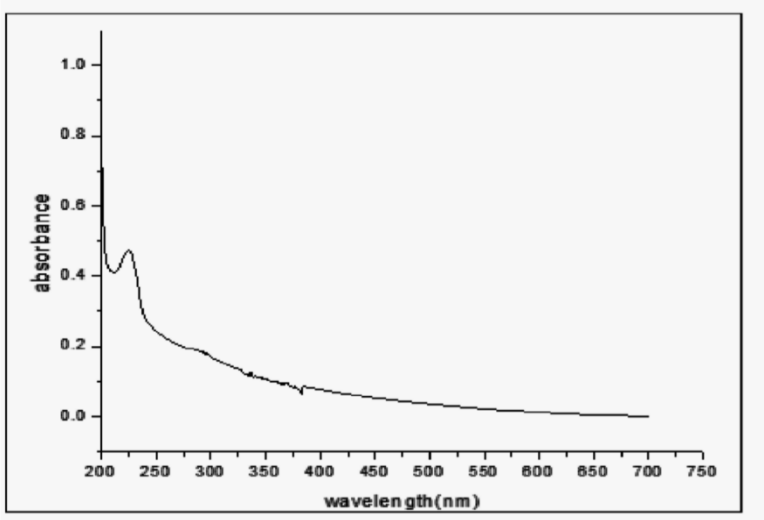


Figure 14: UV-Vis spectra of the GO-Lysozyme conjugate.

4.6.2 Fluorescence emission spectra measurements of GO-LYZ conjugate

The fluorescence emission spectra of lysozyme and with GOns was taken within the spectral region between 300 and 380 nm. The fluorescence emission spectra were taken using excitation maxima of 280 nm. It was observed that tryptophan florescence intensity was reduced in the presence of GOns. This shows that after binding of GOns to protein makes some conformational changes in the protein.

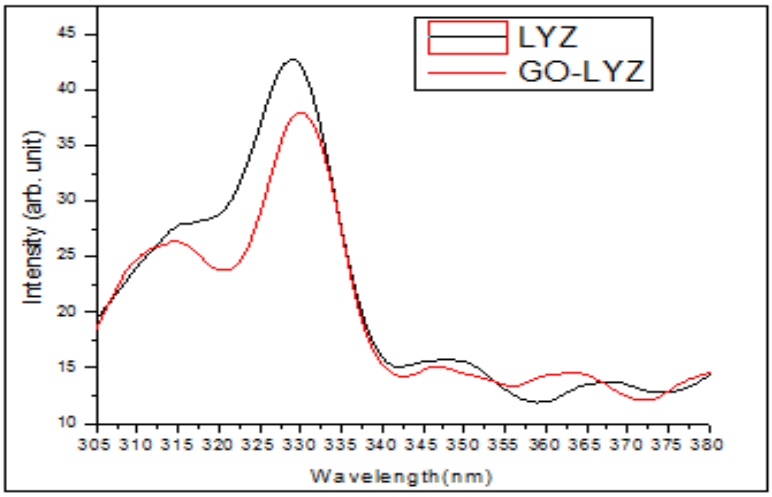


Figure15: Emission Fluorescence spectrum of lysozyme & GOns-LYz.

4.6.3 FESEM analysis of GOns-LYZ conjugate

Lysozyme react with GOns and surrounds the GOns and makes a coating. Lysozyme conjugates shows round shape structure around 300 to 400nm.Due to adsorption of protein on GOns, it covers the sheets and forms round shaped structure.

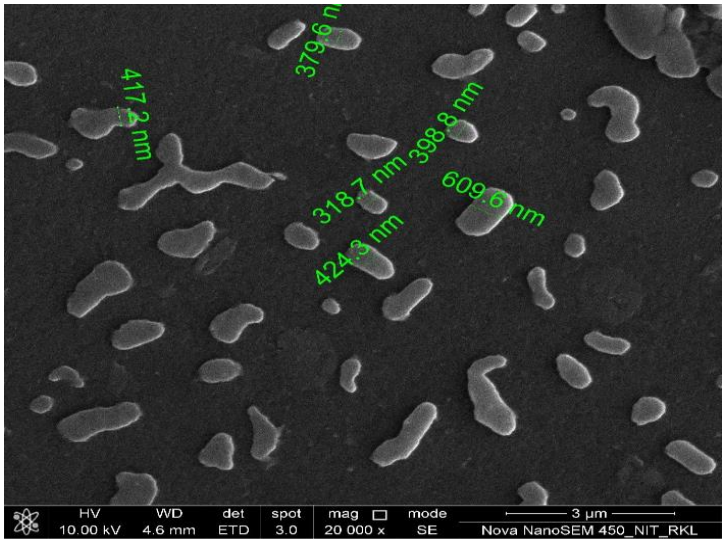
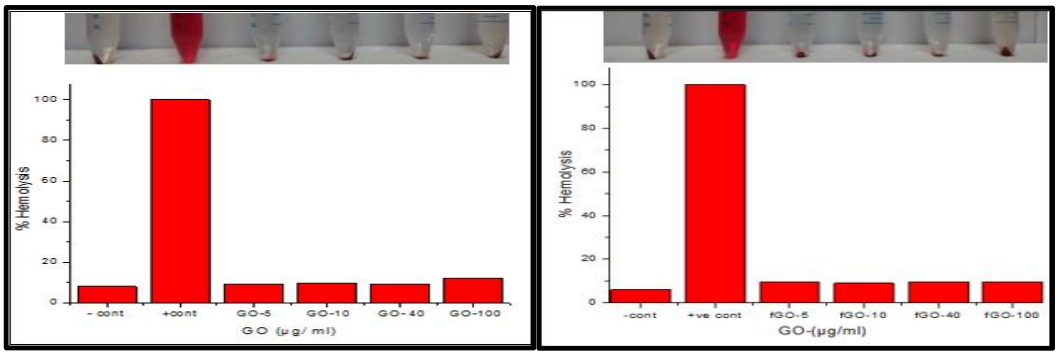


Figure16: FESEM image of GOns-LYZ conjugate.

4.7 Hemolysis assay of GOns-LYZ conjugate (1:1) and (1:2)

For GOns-LYZ conjugate hemolysis is increased on increasing the concentration of conjugates. For GOns-LYZ (1:1) the maximum hemolysis was 12% and for (1:2) 9% as compared to triton X 100.After



(a) **(b)**

Figure17: Hemolysis assay of Erythrocyte by GOns-LYZ conjugate in (1:1) **(and** (1:2)

(b) Data was taken in trplicates and error was within 3%.

conjugation the GOns surface is modified due to which the oxygen groups are reducing so that there will be less electrostatic attraction and there will be less membrane disruption.

4.8 Effect of GOns-LYZ Conjugates (1:1) and (1:2) on the growth of *P. aeruginosa*

The optical density of *P. aeruginosa* is reduced as the concentration of GO is increased from 50 to 500µg/ ml. There is more decrement in OD when GO-LYZ (1:1) is given compared to GO-LYZ (1:2).Lysozyme has its own antibacterial effect or as well as after conjugation structure of lysozyme may be changed which might be enhancing its antibacterial effect.

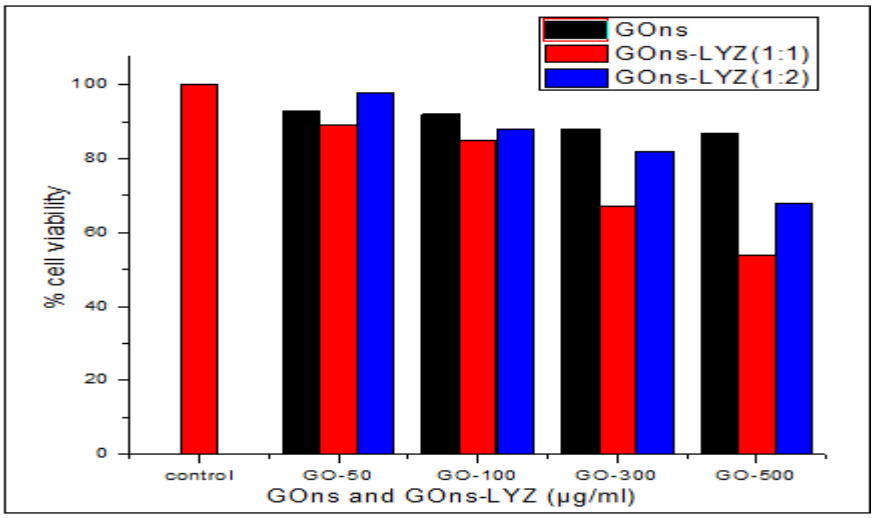


Figure 18: Cell viability assay of GOns-LYZ conjugate of *P.aeruginosa*

and error was withn 3%.

CHAPTER5

Conclusion

In this present investigation GO nanosheets (GOns) were synthesized successfully by pyrolysis of citric acid method and The detail characterization of the GO nanosheets was carried out using UV-Vis spectroscopy, Field emission Scanning Electron Microscopy (FESEM), X-Ray Diffraction (XRD) analysis and Raman Spectroscopy. FESEM image analysis, the average particle size was found to be 200-500nm.Antibacterial effect of GOns was evaluated against three different bacteria like *Escherichia coli*, *Pseudomonas aeruginosa*, and *Lactobacillus Caesi*. The growth study of *Escherichia coli, Pseudomonas aeriginosa and Lactobacillus casei was performed* in the presence of various concentration of GOns and GO-LYZ conjugate to evaluate the growth of bacteria in liquid media. *P. aeruginosa* shows mild antibacterial effect, *E.coli* showed no significant effect in growth while *L.casei* shows enhancement in the growth. Hence GOns can be beneficial for probiotic bacteria and harmful to pathogenic bacteria .GOns conjugation with lysozyme enhances hemocomatibilty as well as antibacterial activity. Therefore in conclusion we state that our prepared GOns-LYZ conjugate van safely be used in antibacterial application as well as the overproduction of probiotics thus can find crucial application in industry.

CHAPTER 6

REFERENCES

Agarwal, S., Zhou, X., Ye, F., He, Q., Chen, G. C., Soo, J.,& Chen, P. (2010). Interfacing live cells with nanocarbon substrates. *Langmuir*, *26*(4), 2244-2247.

Akhavan, O., & Ghaderi, E. (2010). Toxicity of graphene and graphene oxide nanowalls against bacteria. *ACS nano*, *4*(10), 5731-5736.

Akhavan O, Ghaderi E. (2009) Photocatalytic reduction of graphene oxide nanosheets on TiO2 thin film for photoinactivation of bacteria in solar light irradiation.113:20214–20.

Akhavan, O., & Ghaderi, E. (2010). Toxicity of graphene and graphene oxide nanowalls against bacteria. *ACS nano*, *4*(10), 5731-5736.

Allen, M. J., Tung, V. C., & Kaner, R. B. (2009). Honeycomb carbon: a review of graphene. *Chemical reviews*, *110*(1), 132-145.

Allen TM. (2002) Ligand-targeted therapeutics in anticancer therapy.Vol.2:750^63.

Bao, Q.; Zhang, D.; Qi, P. (2011). Synthesis and Characterization of Silver Nanoparticle and Graphene Oxide Nanosheet Composites as a Bactericidal Agent for Water Disinfection.J. Colloid Interface Sci., 360, 463–470.

Cedervall, T.; Lynch, I.; Lindman, S.; Bergg rd, T.; Thulin, E.;Nilsson, H.; Dawson, K. A.; Linse, S. (2007)Understanding the Nanoparticle-Protein Corona Using Methods to Quantify Exchange Rates and Affinities of Proteins for Nanoparticles.Proc. Natl. Acad. Sci. U. S. A., 104, 2050–2055.

Chen, C., Yang, Q. H., Yang, Y., Lv, W., Wen, Y., Hou, P. X., ... & Cheng, H. M. (2009). Self-Assembled Free-Standing Graphite Oxide Membrane.*Advanced Materials*, *21*(29), 3007-3011

Chen, H.; Mu¨ ller, M. B.; Gilmore, K. J.; Wallace, G. G.; Li, D.Mechanically Strong, (2008) Electrically Conductive, and Biocompatible Graphene Paper. *Adv. Mater.*, *20*,3557–3561

Compton, O. C., & Nguyen, S. T. (2010). Graphene Oxide, Highly Reduced Graphene Oxide, and Graphene: Versatile Building Blocks for Carbon-Based Materials. *small*, *6*(6), 711-723.

Cong, H. P., He, J. J., Lu, Y., & Yu, S. H. (2010). Water-Soluble Magnetic-Functionalized Reduced Graphene Oxide Sheets: In situ Synthesis and Magnetic Resonance Imaging Applications. *Small*, *6*(2), 169-173.

Das, M. R., Sarma, R. K., Borah, S. C., Kumari, R., Saikia, R., Deshmukh, A. B.& Boukherroub, R. (2013). The synthesis of citrate-modified silver nanoparticles in an aqueous suspension of graphene oxide nanosheets and their antibacterial activity. *Colloids and Surfaces B: Biointerfaces*, *105*, 128-136.

Dastjerdi, R., Montazer, M., Stegmaier, T., & Moghadam, M. B. (2012). A smart dynamic self-induced orientable multiple size nano-roughness with amphiphilic feature as a stain-repellent hydrophilic surface. *Colloids and Surfaces B: Biointerfaces*, *91*, 280-290.

Dell'Orco, D., Lundqvist, M., Oslakovic, C., Cedervall, T., & Linse, S. (2010). Modeling the time evolution of the nanoparticle-protein corona in a body fluid.*PLoS One*, *5*(6), e10949.

Fang, M., Chen, J. H., Xu, X. L., Yang, P. H., & Hildebrand, H. F. (2006). Antibacterial activities of inorganic agents on six bacteria associated with oral infections by two susceptibility tests. *International Journal of Antimicrobial Agents*, *27*(6), 513-517.

Feng, L., Zhang, S., & Liu, Z. (2011). Graphene based gene transfection.*Nanoscale*, *3*(3), 1252-1257.

.

Gangadharan, D., Harshvardan, K., Gnanasekar, G., Dixit, D., Popat, K. M., & Anand, P. S. (2010). Polymeric microspheres containing silver nanoparticles as a bactericidal agent for water disinfection. *water research*, *44*(18), 5481-5487.

Hu WB, Peng C, Luo WJ, et al. (2010) Graphene-based antibacterial paper. ACS Nano. ; 4:4317–4323

Jianfeng Shen, Min Shi, Bo Yan, Hongwei Ma, Na Li, Yizhe Hu, Mingxin Ye.81, (2010) covelent attaching protein to graphene oxide via diimide activated amidation 434–438

Jung, W. K., Koo, H. C., Kim, K. W., Shin, S., Kim, S. H., & Park, Y. H. (2008). Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli. *Applied and environmental microbiology*, *74*(7), 2171-2178.

Krätschmer, W., Lamb, L. D., Fostiropoulos, K., & Huffman, D. R. (1990). C60: a new form of carbon. *Nature*, *347*(6291), 354-358.

Lai, Y., Zhuang, H., Xie, K., Gong, D., Tang, Y., Sun, L.,& Chen, Z. (2010). Fabrication of uniform Ag/TiO 2 nanotube array structures with enhanced photoelectrochemical performance. *New Journal of Chemistry*, *34*(7), 1335-1340.

Lee, W.; Parpura, V. (2009) Wiring Neurons with Carbon Nanotubes.Front. Neuroeng., 2, 1–3.

Lin, Z.; Liu, Y.; Yao, Y.; Hildreth, O. J.; Li, Z.; Moon, K.; Wong,C. J.(2011) Phys. Chem. Reduced chemically modified graphene oxide for supercapacitor electrodeC, 115, 7120−7125.

Li, L. S., & Yan, X. (2010). Colloidal graphene quantum dots. *The Journal of Physical Chemistry Letters*, *1*(17), 2572-2576.

Li, Q., Mahendra, S., Lyon, D. Y., Brunet, L., Liga, M. V., Li, D., & Alvarez, P. J. (2008). Antimicrobial nanomaterials for water disinfection and microbial control: potential applications and implications. *Water research*, *42*(18), 4591-4602.

Lin, Y., Watson, K. A., Fallbach, M. J., Ghose, S., Smith Jr, J. G., Delozier, D. M., ... & Connell, J. W. (2009). Rapid, solventless, bulk preparation of metal nanoparticle-decorated carbon nanotubes. *Acs Nano*, *3*(4), 871-884.

Li XL, Wang XR, Zhang L, Lee SW, Dai HJ.(2008) Chemically derived, ultrasmooth graphene nanoribbon semiconductors. Science; 319(5867):1229–32.

Liu, Z., Robinson, J. T., Sun, X., & Dai, H. (2008). PEGylated nanographene oxide for delivery of water-insoluble cancer drugs. *Journal of the American Chemical Society*, *130*(33), 10876-10877.

Liu S, Zeng TH, Hofmann M, Burcombe E, Wei J, Jiang R. (2011).Antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide: membrane and oxidative stress. ACS Nano.; 5:96971–96980

Lyon, D. Y., Brunet, L., Hinkal, G. W., Wiesner, M. R., & Alvarez, P. J. (2008). Antibacterial activity of fullerene water suspensions (nC60) is not due to ROS-mediated damage. *Nano letters*, *8*(5), 1539-1543.

Maeda, H. (2010). Tumor-selective delivery of macromolecular drugs via the EPR effect: background and future prospects. *Bioconjugate chemistry*, *21*(5), 797-802.

M. R. Das, R. K. Sarma, R. Saikia, V. S. Kale, M. V. Shelke and P. Sengupta, (2011) Synthesis of silver nanoparticles in an aqueous suspension of graphene oxide sheets and its antimicrobial activity.Colloids Surf., B, , 83, 16–22.

Nel, A., Xia, T., Mädler, L., & Li, N. (2006). Toxic potential of materials at the nanolevel. *Science*, *311*(5761), 622-627.

Park, S., Mohanty, N., Suk, J. W., Nagaraja, A., An, J., Piner, R. D., ... & Ruoff, R. S. (2010). Biocompatible, Robust Free-Standing Paper Composed of a TWEEN/Graphene Composite. *Advanced Materials*, *22*(15), 1736-1740.

Ponomarenko, L. A., Schedin, F., Katsnelson, M. I., Yang, R., Hill, E. W., Novoselov, K. S., & Geim, A. K. (2008). Chaotic Dirac billiard in graphene quantum dots. *Science*, *320*(5874), 356-358.

Qi, X. Y., Ma, C. B., Nai, S. M. L., Cheng, C. K., Zhang, H., & Wei, J. (2012, November). Enhanced Mechanical Properties of Poly (Vinyl Alcohol) Nanofibers With Molecular Level Dispersed Graphene. In *ASME 2012 International Mechanical Engineering Congress and Exposition* (pp. 375-379). American Society of Mechanical Engineers.

Qi, X., Poernomo, G., Wang, K., Chen, Y., Chan-Park, M. B., Xu, R., & Chang, M. W. (2011). Covalent immobilization of nisin on multi-walled carbon nanotubes: superior antimicrobial and anti-biofilm properties. *Nanoscale*, *3*(4), 1874-1880.

Ruparelia, J. P., Chatterjee, A. K., Duttagupta, S. P., & Mukherji, S. (2008). Strain specificity in antimicrobial activity of silver and copper nanoparticles.*Acta Biomaterialia*, *4*(3), 707-716

Ruiz, O. N., Fernando, K. S., Wang, B., Brown, N. A., Luo, P. G., McNamara, N. D.& Bunker, C. E. (2011). Graphene oxide: a nonspecific enhancer of cellular growth. *ACS nano*, *5*(10), 8100-8107.

Santos, C. M., Tria, M. C. R., Vergara, R. A. M. V., Cui, K. M., Pernites, R., & Advincula, R. C. (2011). Films of Highly Disperse Electrodeposited Poly (N-vinylcarbazole)–Graphene Oxide Nanocomposites. *Macromolecular Chemistry and Physics*, *212*(21), 2371-2377.

Schedin, F.; Geim, A. K.; Morozov, S. V.; Hill, E. W.; Blake, P.;Katsnelson, M. I.;Novoselov, K. S. Nat (2007) Detection of individual gas molecules adsorbed on graphene.Mater., 6, 652−655.

Shen, J., Zhu, Y., Yang, X., Zong, J., Zhang, J., & Li, C. (2012). One-pot hydrothermal synthesis of graphene quantum dots surface-passivated by polyethylene glycol and their photoelectric conversion under near-infrared light.*New Journal of Chemistry*, *36*(1), 97-101.

Shi, L., Hernandez, B., & Selke, M. (2006). Singlet oxygen generation from water-soluble quantum dot-organic dye nanocomposites. *Journal of the American Chemical Society*, *128*(19), 6278-6279.

Sun, X., Liu, Z., Welsher, K., Robinson, J. T., Goodwin, A., Zaric, S., & Dai, H. (2008). Nano-graphene oxide for cellular imaging and drug delivery. *Nano research*, *1*(3), 203-212.

Y.W. Zhu, S. Murali, W.W. Cai, X.S. Li, J.W. Suk, J.R. Potts, R.S. Ruoff, (2010) Adv. Mater.Graphene and graphene oxide: synthesis, properties, and applications.22,3906.

Zhang, J.L. et al. (2010) Graphene oxide as a matrix for enzyme immobilization. Langmuir 26, 6083–6085

Zhang, Y. B.; Ali, S. F.; Dervishi, E.; Xu, Y.; Li, Z. R.; Casciano, D.; Biris, A. S.(2010) Cytotoxicity Effects of Graphene and Single-Wall Carbon Nanotubes in Neural Phaeochromocytoma-Derived PC12 Cells. ACS Nano, 4, 3181–3186.